

Amendments to the Specification

Please replace the paragraph at page 1, line 8 through line 11 with the following amended paragraph and move the paragraph to be the first paragraph of the Specification.

Related Applications

This application claims priority to ~~(the benefit of) U.S. Provisional Application No. U.S.S.N. 60/211,287, entitled, "Sex Non-Specific Methods and Compositions for Increasing Bone Mass", filed on June 13, 2000 and U.S.S.N. U.S. Provisional Application No. 60/274,373, entitled "Methods of Dissociating Nongenotropic from Transcriptional Activity of Steroid Receptors", filed on March 8, 2001.~~

Please replace the paragraph at page 16, line 23 through page 17, line 8 with the following amended paragraph:

Figure 1 FIGS. 1A-1E is a series of graphs demonstrating the control of osteoblast and osteocyte apoptosis by sex steroids *in vivo* and *in vitro*. **FIG. 1A.** Prevalence of osteoblast and osteocyte apoptosis following ovariectomy or orchidectomy in mice. Bars are means \pm SD of 4-5 animals per group. * $p < 0.05$. **FIG. 1B.** Calvarial osteoblasts were treated for 1 hour with 10^{-11} M to 10^{-6} M of E_2 or DHT followed by 6 hours treatment with the pro-apoptotic agent etoposide (50 μ M). The percentage of dead cells was quantified by trypan blue staining. Etoposide-induced apoptosis in the absence of steroids was set at 100%. Bars indicate means \pm SD of triplicate determinations, * $p < 0.05$ for E_2 - or DHT-treated cultures *versus* vehicle using separate one-way ANOVAs for each treatment. **FIGS. 1C and 1D.** Calvaria-derived osteoblasts or MLO-Y4 osteocytic cells were treated with 10^{-8} M E_2 or DHT, and apoptosis was induced by addition of etoposide, dexamethasone (10^{-7} M), or $TNF\alpha$ (1 nM). The percentage of dead cells was quantified as in **FIG. 1B**. Results were analyzed by two-way ANOVA. * $p < 0.05$ when comparing pretreatments within each agent group and each agent *versus* vehicle for a given pretreatment. **FIG. 1E.** OB-6 osteoblastic cells incorporated into collagen gels were treated with different concentrations of E_2 or DHT for 24 hours. A total of 200 cells were evaluated in randomly selected fields. In vehicle controls, 32.3 ± 2.5 % of the cells were apoptotic. * $p < 0.05$ for E_2 - or DHT-treated cultures *versus* vehicle using separate one way ANOVAs for each

treatment.

Please replace the paragraph at page 17, lines 9 through 26 with the following amended paragraph:

Figure 2 FIGS. 2A-2B is a series of graphs showing that the anti-apoptotic effects of E₂ or DHT are inhibited by both ER or AR antagonists and can be mediated via either the ER (α or β) or the AR. **A.** Calvarial osteoblasts were pre-treated with the ER antagonist ICI 182,780 (10^{-7} M) or with the AR antagonist flutamide (10^{-7} M) for 30 min, followed by incubation with 10^{-8} M E₂ or DHT for 1 hour. Subsequently, etoposide was added and apoptotic cells were quantified after 6 hours as in **Figure FIG. 1B**. Results are expressed as percentage of etoposide-induced apoptosis in the absence of steroids which was set at 100%. Bars indicate means \pm SD of triplicate determinations, * $p < 0.05$ *versus* vehicle, by ANOVA. **FIG. 2B.** HeLa cells were transiently transfected with either ER α , ER β , AR, VDR, or RXR expression vectors or with the empty vector (ev), along with a Nuc-EGFP expression vector. Sixteen hours after transfection, cells were treated with the indicated concentrations of E₂, DHT, or 1,25-dihydroxy-Vitamin D₃ (1,25D₃) for 1 hour followed by 6 hours treatment with etoposide. Apoptotic cells were identified by examining the nuclear morphology of 200-500 transfected (fluorescent) cells. Etoposide-induced apoptosis in cells cultured in the absence of steroids (-) was set at 100%. Results are expressed as percentage of etoposide-induced apoptosis (mean \pm SD). * $p < 0.05$ *versus* cells treated with etoposide in the absence of steroids by ANOVA.

Please replace the paragraph at page 17, line 27 through page 18, line 10 with the following amended paragraph:

Figure 3 FIGS. 3A-3B shows the localization of the anti-apoptotic activity of the ER α to the E (ligand binding) domain and elimination by nuclear targeting. **FIG. 3 A.** HeLa cells were co-transfected with expression vectors for Nuc-EGFP and various ER α mutants, and then treated for 1 hour with 10^{-8} M E₂ followed by 6 hours treatment with etoposide. **FIG. 3 B.** The full length ER α was fused to the non-targeted ECFP (ER α -ECFP). The E domain of the ER α was fused to either non-targeted ECFP (E-ECFP), membrane targeted ECFP (E-Mem-ECFP) or nuclear targeted ECFP (E-Nuc-ECFP). Each of these fusion proteins was expressed in HeLa cells

along with a nuclear targeted red fluorescent protein (Nuc-ERFP) and their subcellular distribution was analyzed by epifluorescence microscopy. Left and middle panels depict photomicrographs of the same cell(s) obtained with either cyan or red filter sets, respectively; bar = 15 μ m. Nuc-ERFP was used in this experiment to quantify apoptosis because its emission can be readily distinguished from that of the cyan fusion proteins. Transfected cells were treated for 1 hour with 10^{-8} M E_2 or vehicle followed by 6 hours treatment with etoposide. Apoptosis in both **FIGS. 3A** and **3B** was quantified as in **Figure FIG. 2**. Bars indicate means \pm SD of triplicate determinations. * $p < 0.05$ versus cells not treated with etoposide by ANOVA.

Please replace the paragraph at page 18, lines 11 through 28 with the following amended paragraph:

Figure 4 FIGS. 4A-4E shows that the anti-apoptotic activity of the ER and AR is mediated via a Src/Shc/ERK signaling pathway. **FIG. 4A**. MLO-Y4 cells were incubated for 2, 5, or 15 min with E_2 or DHT or for 25 min with PD98059 (50 μ M) or PP1 (10 μ M) followed by 5 min with E_2 or DHT. Cell lysates were obtained and proteins were analyzed by Western blot analysis using anti-ERK1/2 or anti-phospho-ERK1/2 (p-ERK1/2) antibodies. For the determination of apoptosis, cells were incubated with vehicle, PD98059 or PP1 30 min prior to the addition of E_2 or DHT. After 30 min, etoposide was added and apoptosis was assayed 6 hours later as in **Figure FIG. 1B**. **FIG. 4B**. The effect of E_2 or DHT on apoptosis was compared in embryonic fibroblasts from Src+/+ versus Src-/- mice, as described in **Figure FIG. 1B**. RT-PCR was performed as described in the experimental procedures. ~~C-E~~ **FIGS. 4C - 4E**. HeLa cells were transfected with Nuc-EGFP and with expression vectors encoding the full length ER α or its E domain, or the AR alone (~~C~~ **FIG. 4C, left panel**) or together with a dn MEK (~~C~~ **FIG. 4C, right panel**); a Src mutant lacking the kinase activity (Src K⁻), a Src mutant lacking the SH2 domain (Src Δ SH2), or a Src mutant lacking the SH3 domain (Src Δ SH3) (~~D~~ **FIG. 4D**); or with ER α together with wt Shc, or the dn Shc mutants Y239F/Y240F/Y317F (Shc FFF), Y317F (Shc YYF) or Y239F/Y240F (Shc FFY) (~~E~~ **FIG. 4E**). Bars represent means \pm SD of triplicate determinations. * $p < 0.05$ versus cells cultured without steroids, by ANOVA.

Please replace the paragraph at page 18, line 29 through page 19, line 14 with the following amended paragraph:

Figure 5 FIGS. 5A-5C demonstrates the dissociation of ER α -mediated transcriptional *versus* anti-apoptotic actions and ER α -mediated *versus* ER β -mediated effects using peptides. **FIG. 5A.** HeLa cells were transiently transfected with the ER α expression vector alone or together with a vector carrying a fusion of the α -II peptide with the GAL4 DNA binding domain driven by the SV40 promoter. In addition, cells were transfected with ERE-luc or IL-6-luc for the transcription studies; or with EGFP-nuc for the apoptosis experiments. Bars represent the mean \pm SD of triplicate determinations and indicate fold-stimulation of ERE-luc, or fold-inhibition of IL-6-luc, or % of transfected cells with apoptotic features in vehicle (E_2 -) or 10^{-8} M E_2 (E_2 +). * $p < 0.05$ *versus* vehicle treated cells, by ANOVA. **FIG. 5B.** HeLa cells were transfected with ER α or ER β alone or together with expression vectors carrying 2X293 or 2XF6 peptides. Bars represent mean \pm SD of triplicate determinations of the activity (ERE-luc, IL-6-luc, or anti-apoptosis) in response to E_2 (10^{-8} M) in cells transfected with the indicated receptor and peptide *versus* cells transfected with the receptor alone. E_2 -induced activity in cells carrying the receptor, but not the peptide, was designated as 100%. * $p < 0.05$ *versus* cells transfected with the receptor alone, by ANOVA. **FIG. 5C.** HeLa cells were transfected with a vector carrying the GRIP peptide alone or together with ER α . Bars represent the mean \pm SD of triplicate determinations. * $p < 0.05$ *versus* vehicle treated cells, by ANOVA.

Please replace the paragraph at page 19, lines 15 through 24 with the following amended paragraph:

Figure 6 FIGS. 6A-6B demonstrates the dissociation of nongenotropic from genotropic activity of the ER with synthetic ligands. **FIG. 6A.** For C3-mediated transcription, HeLa cells were transfected with C3-luc and ER α , and were treated with the indicated concentrations of an estren or a pyrazole. Bars represent the mean \pm SD of the relative luciferase units (RLU) normalized for β -galactosidase activity. $p < 0.05$ *versus* cells transfected with empty vector (ev), by ANOVA. The anti-apoptotic activity of the estren and the pyrazole was evaluated in calvaria-derived osteoblasts as described in **Figure FIG. 1B**. * $p < 0.05$ *versus* cells not treated with etoposide by ANOVA. **FIG. 6B.** MLO-Y4 cells were incubated for 5 min with the indicated

concentrations of the different ligands and ERK phosphorylation was assessed as in **Figure FIG. 4A**.

Please replace the paragraph at page 19, line 25 through page 20, line 12 with the following amended paragraph:

Figure FIG. 7 shows a proposed model for ligand-induced dissociation of anti-apoptotic from classical genotropic activity of sex steroid receptors. The three cartoons depict conformational states of the receptor protein prior to and following interaction with the ligands, that are required to effect either the genotropic or the anti-apoptotic responses. The inactive unligated receptor is depicted in the middle in white. The change in conformation induced by interaction with a ligand that preferentially triggers transcriptional activity (e.g., the pyrazole of **Figure FIG. 6**) is depicted in the right in blue right downward diagonal crosshatching. The change in conformation induced by interaction with a ligand that preferentially triggers the anti-apoptotic activity of the receptor (e.g., the estren of **Figure FIG. 6**) is depicted in magenta the left in horizontal lines. White green circle and green white diamond represent the two ligands; please note the perfect and imperfect fit within the binding pocket, respectively. Association (k_a) and dissociation (k_d) rates of the two different types of ligands for the receptor are depicted by different width and length arrows. Ligands such as E_2 will of course induce both conformations. Although in the anti-apoptotic model a direct contact between the receptor and Src is shown, it is possible that adapter protein(s) may bridge the interaction between the two molecules. Corresponding energy levels of the receptor protein in the inactive unligated state (broken line), progressing either to the genotropic conformation (right blue line) or the anti-apoptotic conformation (left magenta line) are shown in the bottom. The discontinuous vertical axis in the energy diagram is meant to indicate a larger than scale difference in energy requirements between the two activities.

Please replace the paragraph at page 20, lines 13 through 14 with the following amended paragraph:

Figure FIG. 8 is a diagram of two transgene constructs that are co-introduced into the same mouse for Dox-induced expression of the α II and 293 peptides.

Please replace the paragraph at page 20, lines 15 through 25 with the following amended paragraph:

Figure FIG. 9A is a diagram illustrating the generation and functional characterization of the Dox-inducible system for osteoblast-specific expression of Cre recombinase. Activation of the ROSA26 locus by Cre under the control of the OG-2 promoter. “SA” indicates a splice acceptor site of the original ROSA26 locus. **Figure FIG. 9B** is gel showing Dox-induced activation of Cre expression in vitro. Ob-6 cells were transiently transfected with the Tet-OG2-Cre constructs and were then treated with 0.5mg/ml Dox or vehicle for 24 hours. Cells were harvested and Cre expression was assessed by RT-PCR analysis. Lane 1 contains size markers. Lane 2 represents cells treated with Empty Vehicle plus Dox. Lane 3 represents cells containing OG-2-rtA-Cre without Dox. Lane 4 represents cells containing OG-2-rtA-Cre with Dox. Lane 4 contains the OG-2rtA-Cre plasmid induced with Dox.

Please replace the paragraph at page 20, lines 26 through 27 with the following amended paragraph:

Figure FIG. 10 is a diagram illustrating the targeting of floxed exon 3 into a 86 Kb genotrophic locus containing exons 3 and 4 of the mouse ER α gene.